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Identification of Amylase-Producing Thermophilic Bacteria through 16sRNA Gene Analysis: MGH3 Strain

Ruth Rize Paas Megahati S[1]

[1] Medical Laboratory Engineering Study Program, Politeknik Kesehatan Kesuma Bangsa, Bandar Lampung, Lampung, Indonesia.

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Corresponding Author:
Ruth Rize Paas Megahati S
megahati71@gmail.com

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Abstract: Thermostable amylase plays a role in the industrial sector because it is resistant to high temperatures, has high enzyme activity in various parameters and is economical in production costs. The thermostable amylase used in industry is stable and thermo activity. Bacteria that produce thermostable amylase can come from hot springs with temperatures above 80°C, such as Semurup hot springs, Kerinci, Jambi province, Indonesia. Semurup hot spring (Jambi) has a temperature of 82°C and a pH of 8.6. This causes variations in the content of organic, inorganic, and mineral compounds in the Semurup hot springs. The varied mineral content causes thermophilic bacteria to survive and the genetic diversity of thermophilic bacteria varies. This study aimed to molecularly identify thermostable amylase-producing thermophilic bacteria by analyzing the 16sRNA gene. Identification of bacteria was carried out by analyzing the 16S rRNA gene of thermophilic bacteria, such as DNA Isolation, DNA Amplification, 16S rRNA Gene Analysis, Alignment, and Phylogenetic Tree. There were 4 different bases between the bacterial isolates MGH3 and Bacillus licheniformis ATCC 14580, namely bases no. 87, 120, 967, and 1374. The difference of 4 bases in the two bacterial isolates already shows that the two isolates have a close kinship. So, most likely the MGH3 bacterial isolate is Bacillus licheniformis strain MGH3.

Keywords: Bacterial identification; Hot spring; Thermophilic bacteria; 16sRNA gene

Introduction

Bacteria can live in extreme environmental conditions, such as environments, acids, bases, and low and high temperatures. Bacteria that can live at high temperatures are known as thermophilic bacteria (Rakhmawati et al., 2021). Thermophilic bacteria can live at temperatures above 45ºC (Lischer et al., 2020). Thermophilic bacteria produce various kinds of thermophilic enzymes, such as protease, lipase, cellulase, and amylase. Amylase derived from thermophilic bacteria is thermostable (Indriati et al., 2018). Thermostable amylase is an enzyme that catalyzes the hydrolysis of α-1,4-glycosidic bonds in starch to glucose (Indriati et al., 2018).

Thermostable amylase plays a role in the industrial sector because it is resistant to high temperatures, has high enzyme activity in various parameters, and is economical in production costs (Indriati et al., 2018). Thermostable amylase is used commercially in the leather tanning, bioethanol, textile, detergent, and food industries (Indriati et al., 2021). Thermostable amylase can be obtained from thermophilic bacteria originating from geothermal sources, volcanic areas, and hot springs.

The thermostable amylase used in industry is stable and has thermo activity (Yassin et al., 2021). Bacteria that produce thermostable amylase can come from hot springs with temperatures above 80°C, such as Semurup hot springs, Kerinci, Jambi province, Indonesia. Semurup hot spring (Jambi) has a temperature of 82°C and a pH of 8.6. This causes variations in the content of organic, inorganic, and mineral compounds in the Semurup hot springs. The varied mineral content causes thermophilic bacteria to survive and the genetic diversity of thermophilic bacteria varies. This study aimed to molecularly identify thermostable amylase-

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producing thermophilic bacteria by analyzing the 16sRNA gene.

Method

Identification of bacteria was carried out by analyzing the 16S rRNA gene of thermophilic bacteria.

DNA Isolation

The bacterial genomic DNA was isolated by rinsing the bacterial suspension using 500 µl TE buffer 1x and centrifuging at 13,000 rpm for 5 minutes. The resulting pellet was suspended with 50 µl TE buffer 1x, 300 µl extraction buffer, then mixed using a vortex for 5 minutes. A total of 150 µl 3M sodium acetate was added to the suspension and incubated at room temperature for 10 minutes. Then the suspension was centrifuged for 5 minutes at 13,000 rpm. The supernatant was transferred into a new microtube and added with the same volume of isopropanol (1:1). The mixture was briefly inverted and centrifuged at 13,000 rpm for 10 minutes. The resulting pellet was washed with 70% ethanol and then centrifuged for 1 minute at 13,000 rpm. The resulting genomic DNA or pellet was air-dried and resuspended with 50 µl of TE buffer 1x (Wright et al., 2017; Barnes et al., 2014)

DNA Amplification

16S rRNA gene amplification was performed using GoTaq (Promega) with primers 27F (5'-AGAGTTTGATCCTGGTCCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR conditions were adjusted for 30 cycles with predenaturation temperature 94°C for 5 minutes, denaturation 94°C for 45 seconds, annealing 50°C for 45 seconds, extension 72°C for 45 seconds, and post extension 72°C. The sequencing results were then processed using the Crustal X, Bioedit, and MEGA 5 programs (Giangacomo et al., 2021; Rosselli et al., 2016).

16S rRNA Gene Analysis

The 16S rRNA gene was sequenced at BLAST and compared with other bacterial 16S rRNA genes deposited at Genbank (Indriati et al., 2021).

Alignment

Alignment of sequence data is carried out with the Crustal X program. Alignment begins with inputting data into the Crustal X file in the form of Fasta and then alignment is performed. The alignment results are then edited with the Bioedit program (Thompson, 1997; Ishaq et al., 2019).

Phylogenetic Tree

Phylogenetic trees are made based on DNA sequences using software programs. The software program is based on several statistical methods, namely the Distance, Parsimony, and Likelihood methods. In the Distance method, the evolutionary distance is calculated for all pairs of taxa and a phylogenetic tree is created based on the relationship between the distance values. Parsimony maxima based on the smallest number of nucleotide substitutions describe the overall evolutionary process to create a phylogenetic tree. The phylogenetic tree is best when the number of substitutions is small. Maximum likelihood is based on calculating the number of possible site variation patterns produced by the substitution process and the observed base frequencies (Smith et al., 2018; Win Win Mar et al., 2020).

Result and Discussion

Isolation of the genomic DNA of the MGH3 bacterial isolate showed the presence of DNA bands, which means that the genomic DNA was successfully isolated (Figure 1).

Figure 1. Results of isolation of genomic DNA from MGH3 bacteria

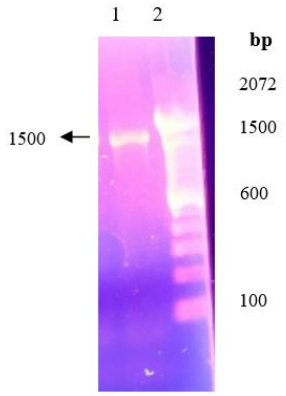

Figure 2. Results of 16S rRNA gene amplification

The genome of the MGH3 bacterial isolate was then amplified and a DNA band of ± 1500 bp was seen (Figure 2). This shows that the PCR conditions were suitable for

amplifying the 16S rRNA gene from MGH3 bacterial isolates.

The sequencing results were compared with the DNA sequences of 11 bacteria deposited at Genbank through the Basic Local Alignment Search Tool (BLAST) program. BLAST results have a similarity value of 95-99%. The value of genetic distance or the difference in nucleotides between isolates (p-distance) is 0.000-1.127. The kinship of 11 bacterial isolates with the MGH3 bacterial isolate showed that the MGH3 bacterial isolate had a close or kinship relationship with Bacillus licheniformis strain ATCC 14580 with a genetic distance value (p-distance = 0.002) and a 99.72% similarity or homology level (Figure 3).

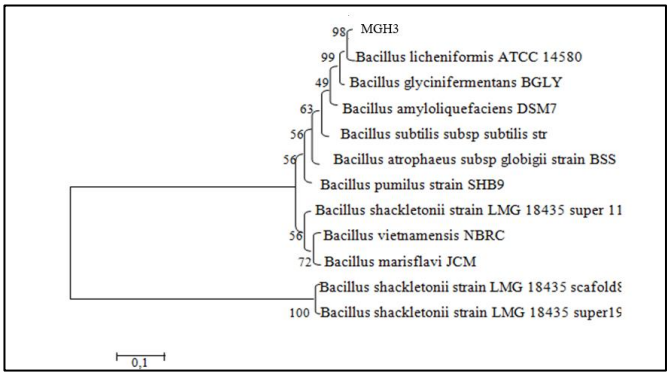

Figure 3. Maximum likelihood phylogenetic tree (Bootstrap method, 1000)

The results of the base sequence analysis using the MEGA 5 program showed that there were 4 different bases between the bacterial isolates MGH3 and *Bacillus licheniformis* ATCC 14580, namely bases no. 87, 120, 967, and 1374. The difference of 4 bases in the two bacterial isolates already shows that the two isolates have a close kinship. So most likely the MGH3 bacterial isolate is *Bacillus licheniformis* strain MGH3. *Bacillus licheniformis* is a bacterium originating from France that can produce antibiotics, biochemical compounds, and various industrial enzymes such as amylase (Ray et al., 2008). Amylase is an enzyme that catalyzes the hydrolysis of α-1,4-glycosidic bonds in starch to glucose (Wahyuni et al., 2020). Amylase is generally a metalloenzyme that requires calcium and chloride ions for activity and maintains the stability and structural integrity of amylase (Nandhany et al., 2020). Amylase will cut the α-1,4 glycosidic bonds in starch molecules (carbohydrates) to form shorter carbohydrate molecules. The results of cutting this enzyme include maltose, maltotriose, and glucose.

In general, *Bacillus licheniformis* ATCC 14850 is a mesophilic bacterium, while MGH3 isolate is a thermophilic bacterium that has been successfully isolated from the Semurup hot springs. The presence of MGH3 bacterial isolates in extreme environments, such as hot springs are due to gene mutations and evolution in Bacillus licheniformis ATCC 14850. So, it can be concluded that MGH3 bacterial isolates are suspected of being a new strain of bacteria, namely *Bacillus licheniformis* strain MGH3.

The results of Fox et al.'s research (Fox et al., 1992; Suzuki et al., 1994) found that *Bacillus globisporus* and *B. psychrophilus* were almost identical based on the 16S rRNA sequences, that is, they only had two consistent base differences in the 16S rRNA sequence but these two species had several differences in several phenotypic characters such as optimum conditions for growth, and can be easily distinguished by DNA-DNA reassociation. Both can be put into different clusters based on the base sequence of the genes encoding pyruvate kinase and alanine dehydrogenase (Kim et al., 2022).

The results of other studies, namely Mycobacterium avium and *M. intracellulare* where both have 16S rRNA gene sequences that are 99.8% identical (Böddinghaus et al., 1990) but both can be distinguished into separate clusters based on the sequences of two protein-coding genes, namely hsp (Drăgan et al., 2016).

The 16S rRNA gene can be used as a molecular marker because this molecule exists in every organism with an identical function in all organisms (Peker et al., 2019). Analysis of the 16S rRNA gene is practical for species definition because this molecule exists in every organism, so a universal primer can be designed for all groups (Johnson et al., 2019). Data on the base sequence of the 16S rRNA encoding gene allows it to be used to construct phylogenetic trees that can show the ancestry and kinship of organisms.

Conclusion

There were 4 different bases between the bacterial isolates MGH3 and Bacillus licheniformis ATCC 14580, namely bases no. 87, 120, 967, and 1374. The difference of 4 bases in the two bacterial isolates already shows that the two isolates have a close kinship. So, most likely the MGH3 bacterial isolate is Bacillus licheniformis strain MGH3

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